Attorney Docket No.: 017170-0006-999
Cam No.: 712576-999009

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Castillo *et al.*
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Art Unit : 1625
Examiner : Covington, Raymond K.
Confirmation No.: 2631

Title : Isolation, Purification And Synthesis Of Procyanidin B2 And Uses Thereof

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF ALAN SNOW UNDER 37 C.F.R. § 1.132

Sir:

I, Alan Snow declare that:

1. I am a co-inventor of the above-identified patent application, and thus am intimately familiar with the subject matter disclosed and claimed in the application.

2. My current position is President and Chief Scientific Officer at ProteoTech, Inc.

3. I have a Ph.D. (1986) in Pathology from Queen's University, Ontario, Canada; M.Sc. (1983) in Anatomy from University of Western Ontario, London, Ontario, Canada and B.S. (1980) in Biology and Chemistry from Bowling Green State University, Bowling Green, Ohio. I have published over 50 publications, over 120 presentations in scientific meetings and I am a co-inventor of over 85 patents.

4. As described in the specification as filed, Examples 7, 8, 14 and 15 demonstrate inhibition/disruption of Aβ amyloid fibrils by isolated and synthetic procyanidin B2 in *in vitro* assays.

Examples 7 (Fig. 13) and 14 (Fig. 24) provide *in vitro* dose dependent data obtained in

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Thioflavin T fluorometry assay. In this assay, Thioflavin T binds specifically to fibrillar amyloid, and this binding produces a fluorescence enhancement at 485 nm that is directly proportional to the amount of amyloid fibrils formed. The higher the fluorescence, the greater the amount of amyloid fibrils formed. As described in Example 7, synthetic procyanidin B2 causes 84.5+/-7.9% disruption/disassembly of preformed Aβ 1-42 fibrils, when used at an Aβ:procyanidin B2 wt/wt ratio of 1:1, and 31.5+/-13.5% disruption when used at an Aβ:procyanidin B2 wt/wt ratio of 1:0.1. As described in Example 14, isolated procyanidin B2 causes 95.5+/-2.7% disruption/disassembly of preformed Aβ 1-42 fibrils, when used at an Aβ:procyanidin B2 wt/wt ratio of 1:1, and 61.6+/-5.8% disruption when used at an Aβ:procyanidin B2 wt/wt ratio of 1:0.1.

Examples 8 (Fig. 14) and 15 (Fig. 25) provide *in vitro* dose dependent data obtained in Congo red binding assay. In this assay, the ability of procyanidin B2 to alter Aβ amyloid binding to Congo red is quantified. Any lowering of the Congo red color in the presence of procyanidin B2 as compared to the Congo red staining of the Aβ amyloid protein in the absence of procyanidin B2 indicates ability of procyanidin B2 to diminish/alter the amount of aggregated and congophilic Ab amyloid. As described in Example 8, synthetic procyanidin B2 causes 46.3+/-4.3% inhibition of Congo red binding to Aβ 1-42 fibrils when used at an Aβ:procyanidin B2 wt/wt ratio of 1:1, and 30.3+/-8.0% inhibition of Congo red binding when used at an Aβ:procyanidin B2 wt/wt ratio of 1:0.1. Isolated procyanidin B2 inhibited Aβ 1-42 fibril binding to Congo red by 36.3+/-4.3% at an Aβ:procyanidin B2 wt/wt ratio of 1:1.

5. Presented herein is *in vivo* data that demonstrates prevention, accumulation, reduction/inhibition of Aβ amyloid by procyanidin B2.

Appendix A, Table 1, provides data indicating prevention of brain amyloid formation/accumulation by procyanidin B (following daily i.p. injections) in APP Transgenic Mice. As seen in Table 1, procyanidin B2 causes 74.2% reduction in Thio S amyloid load and a 74.9% reduction in plaque number as compared to saline-treated APP mice. Further procyanidin B2

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causes 82.9% reduction in Congo red amyloid load, and an 80.8% reduction in plaque number as compared to saline-treated APP mice.

Figure 1, illustrates reduction in amyloid load/plaque number in APP Transgenic Mice after treatment with procyanidin B2 as demonstrated by Thioflavin S Fluorescence. In the Figure, panels A and B are examples of 2 APP transgenic mice treated with saline for 90 days and stained for amyloid plaques in cortex using Thioflavin S. Panels C and D in Figure 1 demonstrate that treatment with procyanidin B2 caused a marked reduction in amyloid load/plaque number in cortex in 2 different APP mice using Thioflavin S fluorescence.

Figure 2, demonstrates the effect of procyanidin B2 treatment on amyloid load in APP Transgenic Mice as shown by Aß Immunofluorescence. In Figure 2, panels A and B are examples of 2 APP transgenic mice treated with saline for 90 days and stained for amyloid plaques in cortex using an Aß antibody (6E10) and immunfluorescence. Panels C and D in the Figure 2 demonstrate the reduction in amyloid load/plaque number after treatment with procyanidin B2 in cortex as shown in 2 different APP mice using Aß immunofluorescence.

Table 2, provides data indicating reduction/inhibition of brain Aß 42/40 levels by procyanidin B2 in APP Transgenic Mice. As seen in Table 2, procyanidin B2 causes 18.5 and 23.9% reduction in insoluble Aß 42 and 40 levels, respectively. For soluble Aß, procyanidin B2 causes 70.4 and 58.9% reduction in Aß 42 and 40 levels, respectively.

Table 3 provides data demonstrating reduction in microgliosis in APP Transgenic Mice after treatment with procyanidin B2. The treatment with procyanidin B2 results in 81.1% reduction in microgliosis (Table 3) and 69% reduction in astrocytosis (not shown).

Figure 3 illustrates improvement in hippocampus-dependent memory (spatial acquisition) as determined by Morris Water Maze Testing. Following 90 days of i.p. injections (50 mg/kg/day) with saline (hAPPtg/saline) or procyanidin B2, APP transgenic mice and non-transgenic littermate controls (Nontg) were tested in a Morris water maze to determine effects on hippocampus-dependent memory (spatial acquisition). As seen in Figure 3, procyanidin B2 treatment

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(hAPPtg/procyanidin B2) causes improvements in hippocampus dependent memory (by 57.8% on day 4 of the invisible platform, and by 57.3% on the 5th day of the invisible platform). This was detected by both path length (meters) and in latency (m/sec). No change in swimming speed (m/sec) between all groups were found. Procyanidin B2 treated APP mice had improvements in spatial acquisition approaching those levels observed in non-transgenic animals (Nontg). On the last day of training, all groups easily found the visible platform demonstrating that all animals had no motor abnormalities.

6. Therefore, based on the evidence presented in the specification and in this declaration, it is my opinion and judgment that the *in vitro* inhibition/disruption of Aβ amyloid fibrils by procyanidin B2 correlates to the *in vivo* prevention, accumulation, reduction/inhibition of Aβ amyloid by procyanidin B2.

I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Declarant's Signature

Alan Snow

Date: 23 January, 2006

Alan Snow, Ph.D.

APPENDIX A

Table 1: Prevention of brain amyloid formation/accumulation by procyanidin B2 in APP Transgenic Mice.

Treatment	Brain Amyloid load (%)		Plaque number (per square mm) %	
	Thio S Fluorescence	Congo red Staining	Thio S Fluorescence	Congo red Staining
Saline	0.99±0.28	0.45±0.13	2.98±0.82	1.56±0.45
Procyanidin B2	0.26±0.11	0.30±0.15	0.77±0.26	0.30±0.15

Table 2: Reduction/inhibition of brain Aß 42/40 levels by procyanidin B2 in APP Transgenic Mice.

Treatment	Aβ42		Aβ40	
	Insoluble (pg/ml)	Soluble (pg/ml)	Insoluble (pg/ml)	Soluble (pg/ml)
Saline	190,588	1637.8	228,920	1530.8
Procyanidin B2	155,247	485.07	174,157	629.3

Table 3: Reduction in microgliosis in APP Transgenic Mice after treatment with procyanidin B2

Treatment	% Area MHC-II immunostained sections
Saline	2.10±0.77
Procyanidin B2	0.39±0.27

APPENDIX B

Figure 1. Reduction in amyloid load/plaque number in APP Transgenic Mice after treatment with procyanidin B2 as demonstrated by Thioflavin S Fluorescence

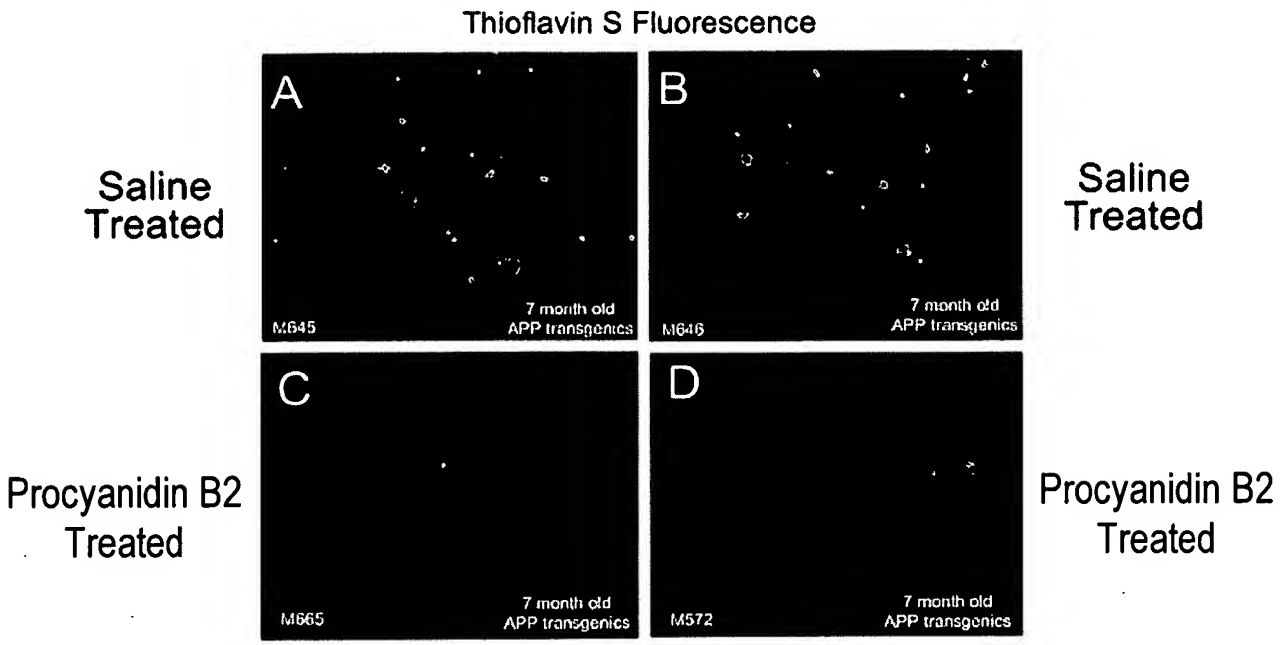

Figure 2: The effect of procyanidin B2 treatment on amyloid load in APP Transgenic Mice as shown by Aß Immunofluorescence.

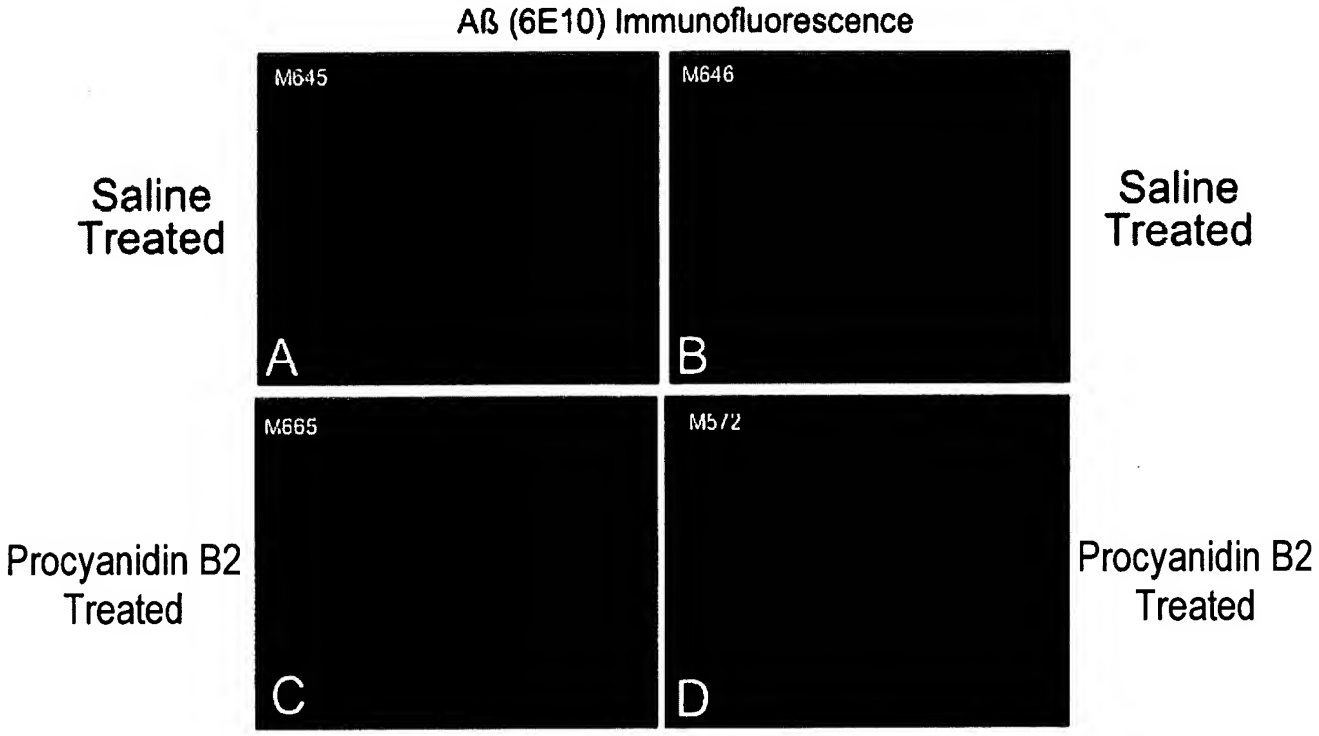

Figure 3: Improvement in hippocampus-dependent memory (spatial acquisition) as determined by Morris Water Maze Testing.